

Proenkephalin Is a Nuclear Protein Responsive to Growth Arrest and Differentiation Signals

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Abstract. Neuropeptide precursors are traditionally viewed as molecules destined to be cleaved into bioactive peptides, which are then released from the cell to act on target cell surface receptors. In this report we demonstrate nuclear localization of the enkephalin precursor, proenkephalin, in rodent and human embryonic fibroblasts (Swiss 3T3 and MRC-5 cells) and in rodent myoblasts (C2C12 cells). Nuclear proenkephalin, detected by immunofluorescence with a panel of anti-proenkephalin monoclonal antibodies, is distributed predominantly in three patterns. Selective abolition of these patterns with salt, nuclease, or methanol is associated with liberation of immunoprecipitable proenkephalin into the extraction supernatant. Proenkephalin antigenic domains, mapped using phage display libraries and synthetic peptides, are differentially revealed in the three distribution patterns. Selective epitope revelation may reflect different conformational forms of proenkephalin or its existence in complexes with other nuclear proteins, forms which therefore have different biochemical associations with the nuclear sub-

structure. In fibroblast cell populations in transition to growth arrest, nuclear proenkephalin responds promptly to mitogen withdrawal and cell-cell contact by transient, virtually synchronous unmasking of multiple antigenic domains in a fine punctate distribution. A similar phenomenon is observed in myoblasts undergoing differentiation. The acknowledgment of growth arrest and differentiation signals by nuclear proenkephalin suggests its integration with transduction pathways mediating these signals.

To begin to address the mechanism of nuclear targeting, we have transfected mutated and nonmutated proenkephalin into COS (African green monkey kidney) cells. Nonmutated proenkephalin is localized exclusively in the cytoplasm; however, proenkephalin mutated at the first ATG codon, or devoid of its signal peptide sequence, is targeted to the nucleus as well as to the cytoplasm. From this we speculate that nuclear proenkephalin arises from a primary translation product that lacks a signal peptide sequence because of initiation at a different site.

PROENKEPHALIN, the precursor to the opioid pentapeptides Met- and Leu-enkephalin (Hughes et al., 1975), is substantially conserved from *Xenopus* to humans (Comb et al., 1982; Martens and Herbert, 1984). There is accumulating evidence for the involvement of proenkephalin and its derivatives in cellular growth control and differentiation. The proenkephalin gene is transiently expressed in the developing central nervous system and in nonneural tissues of mesodermal origin (Keshet et al., 1989). Proenkephalin mRNA is present at a high level in proliferating neuroepithelioma cells (Verbeeck et al., 1990) and primary chondrocyte cultures (Villiger and Lotz, 1992); differentiation is accompanied by downregulation of proenkephalin expression. There is substantial upregulation of proenkephalin gene transcription upon ac-

tivation of T helper cells (Zurawski et al., 1986). Opioid inhibition of neural cell proliferation (Hauser, 1992) and differentiation (Hauser et al., 1989) has been described. Enkephalin precursor protein exists at significant levels in cell types such as astroglia (Melner et al., 1990; Spruce et al., 1990) and lymphocytes (Roth et al., 1989), which suggests a role for proenkephalin itself.

Proenkephalin has to date only been described to exist in the cytoplasm, usually in a secretory pathway distribution. Cells that release unprocessed proenkephalin, such as astrocytes (Batter et al., 1991), also release processing enzymes (Vilijn et al., 1989), which suggests that extracellular cleavage of proenkephalin may occur. Even if cell surface signaling is occurring via small processed peptides, biological activity may still be regulated through the intact precursor. Delta-like opioid receptors and novel, possibly chromatin-associated, opioid binding sites have recently been localized to the nucleus of neuroblastoma cells (Belcheva et al., 1993), raising the possibility of nuclear sites of opioid action.

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In this paper we describe the localization of proenkephalin within the nucleus of fibroblast and myoblast cell lines. Antigenic domains on the proenkephalin molecule are differentially responsive to changes in growth or differentiation state of the cell population. We also show that transfected proenkephalin in which the normal ATG initiation codon has been mutated, or from which the signal peptide has been deleted, is targeted to the nucleus of COS cells. Our ultimate question is why a neuropeptide precursor, generally viewed as a secretory molecule, has a dual fate as a nuclear protein closely integrated with external signals that mediate changes in growth state, the answer to which may have its basis in evolution.

Materials and Methods

Immunolocalization

Cells were seeded at densities between 0.5 and 10^4 per cm^2 (0.2×10^5 per cm^2 for contact inhibition experiments) either into plastic slide flasks (Nunc, Roskilde, Denmark) or onto polylysine-coated glass coverslips, then fixed and stained after 24 h or longer. Cells were fixed in 4% paraformaldehyde (30 min minimum), blocked, and permeabilized in 10% FCS, 0.2% Tween in PBS; antibody incubations (2 h to overnight) were carried out in blocking buffer; wash steps were in 1% BSA, 0.2% Tween in PBS. Indirect immunofluorescence detection was used (fluorochrome-conjugated second antibodies from Jackson Immunoresearch Laboratories, Avondale, PA). DNA was visualized with propidium iodide at 1 $\mu\text{g}/\text{ml}$ in mounting medium. All microscopy was performed with a Biorad/MRC Lasersharp confocal microscope (Bio-Rad, U.K.).

Nuclear Isolation and Immunoblotting

Subconfluent cells were scraped into cold nuclear isolation buffer (NIB; 10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 3 mM MgCl_2 , 0.5% NP40 plus protease inhibitors: 1 mM benzamide, 1 $\mu\text{g}/\text{ml}$ leupeptin, pepstatin, and aprotinin). Nuclei were released from cells by homogenization using a tight-fitting pestle in a glass-glass homogenizer, then centrifuged through a cushion of 30% sucrose in NIB at 1,000 g for 10 min. The isolated nuclei were resuspended in 10 mM Tris-HCl, pH 7.6, 0.5 M NaCl, sonicated for 1 min, then added to an equal volume of twofold concentrated sample buffer before electrophoresis on a 12% denaturing polyacrylamide gel. Proteins were electroblotted onto nitrocellulose; blocking and antibody incubations were carried out in 5% powdered milk, 0.2% Tween in PBS. A chemiluminescence detection system (ECL; Amersham International, Amersham, UK) was used.

Nuclear Extraction

Pelleted cells were preextracted in cold CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl_2 , 1 mM EGTA, 0.5% Triton) plus RNasin and protease inhibitors (1 mM benzamide, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ aprotinin, pepstatin A, and leupeptin) for 3 min and then centrifuged at 650 g for 5 min. Pellets were then extracted with either salt or nuclease. Salt extraction was carried out with 0.25 M ammonium sulfate, pH 6.8, in CSK buffer without NaCl plus protease inhibitors at 4°C for 5 min. Nuclease extraction was carried out with DNase 1 at 50 $\mu\text{g}/\text{ml}$ in low salt (50 mM NaCl) CSK buffer plus protease inhibitors for 20 min at 25°C, followed by RNase to a final concentration of 25 $\mu\text{g}/\text{ml}$ for 10 min at 25°C. In each case, postextraction pellets (650–1,000 g for 5 min) were subjected to immunoblot and supernatants to immunoprecipitation.

Immunoprecipitation

1 μl of monoclonal antibody ascites fluid was added to 250 μl of extraction

supernatant and incubated on ice for 1 h. 20 μl of Protein G-coupled Sepharose beads (Sigma Chemical Co., St. Louis, MO) at 2 mg/ml in CSK buffer was added, followed by an additional 1 h of incubation. Protein G-Sepharose pellets were washed four times in cold CSK buffer, added to an equal volume of twofold concentrated, boiling, SDS sample buffer, then boiled for 5 min. Samples were clarified by centrifugation, then subjected to denaturing PAGE followed by electroblotting. Blots were probed with a different PE mAb from the precipitating antibody and the immunoprecipitation products revealed using ECL chemiluminescence detection.

Mutagenesis and Transient Transfections into COS Cells

Nonmutated human proenkephalin cDNA incorporating the entire coding region and the two in-frame upstream CTG codons was isolated by PCR from the vector pHPE-9 (Comb et al., 1982) using primers that contained BamHI restriction enzyme cleavage sites to facilitate subcloning: upstream primer (STA3), CTCGGATCCGTGTCTGAACCCGGCTTTCC; downstream primer (STOP1), CTCGGATCCTTAAAATCTCAT-AAATCCTCC. Proenkephalin mutated to remove the signal peptide sequence (ATG Δ sig) was isolated by PCR using an upstream primer (STA4) that would bind to the proenkephalin sequence immediately downstream from the signal sequence and that contained a BamHI restriction enzyme site and an ATG initiation codon: STA4, CTCGGATCC-ATGGAATGCAGCCAGGATTGCGCG; STOP1 was again used as the downstream primer. Site-directed mutagenesis (Transformer Mutagenesis Kit; Clontech, Palo Alto, CA) was used to change the upstream CTG codons to TTG and the first ATG codon to ATC, from which translation could not be initiated. Error-free mutagenesis was confirmed by DNA sequencing.

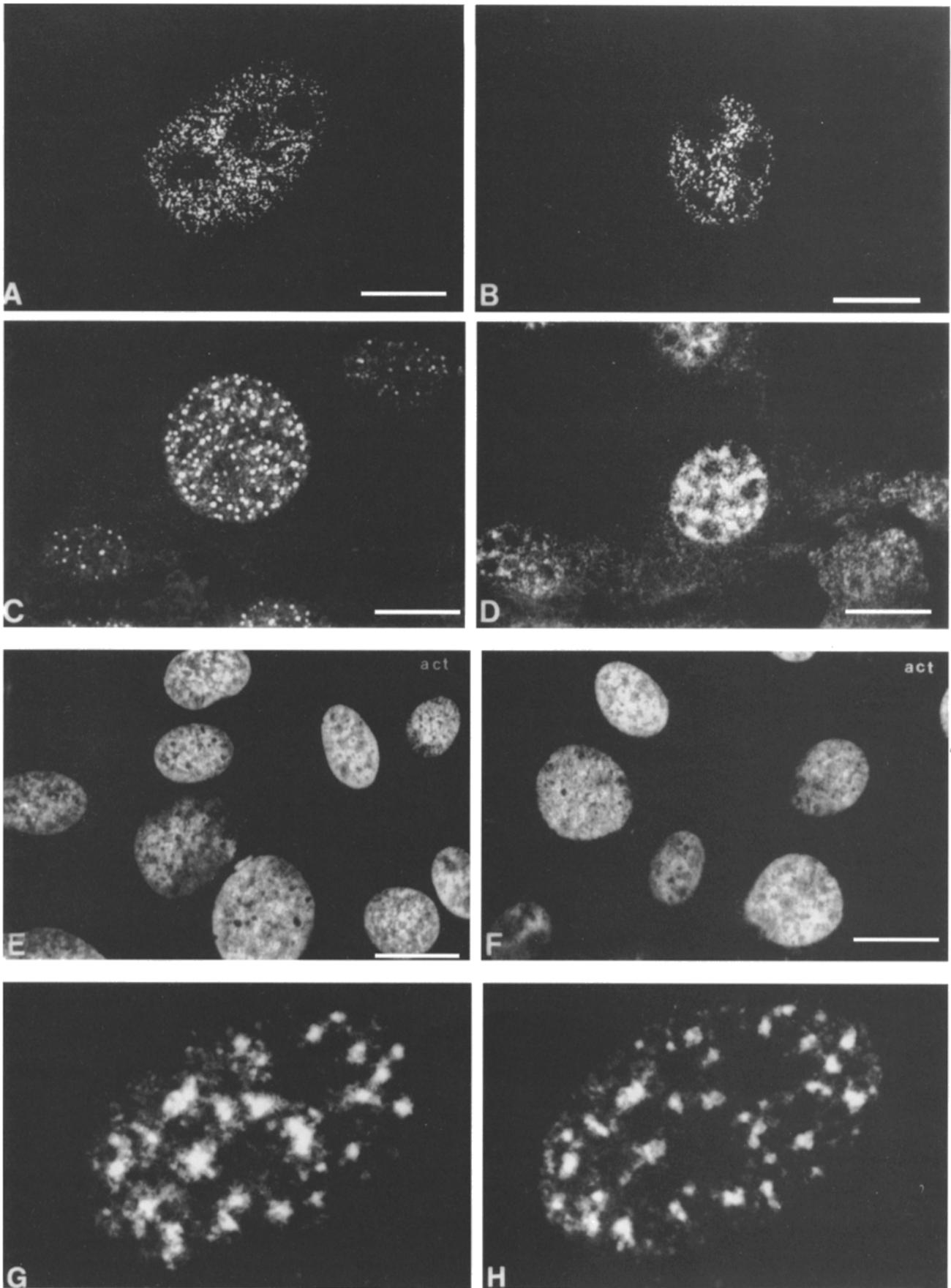
Wild-type and mutant proenkephalin cDNAs were subcloned into the BamHI site of the vector pcDNA3 (Invitrogen, San Diego, CA). pcDNA3:PE constructs were cotransfected into COS cells with pSV β gal (Promega Corp., Madison, WI) by electroporation using a gene pulser (Bio-Rad). Before electroporation, COS cells were grown in 14-cm dishes in DME plus 10% FCS to a density of $3\text{--}4 \times 10^6$ cells per dish. For electroporation they were trypsinised, washed twice in serum-free DME without antibiotics, resuspended in 0.8 ml ice-cold DME, then transferred to chilled electroporation chambers. 10 μg pcDNA3:PE and 10 μg pSV β gal as circular plasmid DNAs were added to each chamber, and the cells and DNA were incubated on ice for 10 min. Control cells were incubated with pSV β gal alone. Electroporation was carried out at 0.25 kV at a capacitance of 500 microfarads. Cells were returned to ice for an additional 10 min, then plated at 5×10^4 cells per milliliter onto polylysine-coated coverslips. Cells were fixed with 4% paraformaldehyde and stained in parallel for proenkephalin and β -galactosidase expression 52 to 65 h later. β -galactosidase expression was assessed by incubating fixed cells with 0.25 mg/ml X-gal in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride at 37°C for 8 h. Proenkephalin immunostaining was performed as described previously.

Results

Subnuclear Localization of Proenkephalin in Multiple Domains

Using a panel of 15 monoclonal antibodies (PE mAbs; PE-1, 2, 13–25) generated to bacterially expressed human proenkephalin fusion polypeptides (Spruce et al., 1988, 1990), we have localized proenkephalin within the nucleus of Swiss 3T3 cells by immunofluorescence microscopy and immunoblotting. Nuclear immunofluorescent staining procedures were performed on paraformaldehyde fixed cells. Fig. 1, A–D, illustrates confocal micrographs of the three

Figure 1. Subnuclear distribution of proenkephalin. Indirect immunofluorescent staining was carried out on exponential cultures of Swiss 3T3 cells. All microscopy was performed with a confocal laser scanning microscope. (A–D) Representative examples of nuclear proenkephalin distributions revealed with different PE mAbs (A and B, PE-1 and PE-2, fine punctate; C, PE-15, coarse punctate; D, PE-16, speckled). (E and F) The change in proenkephalin distributions after actinomycin treatment (E, PE-15; F, PE-16). Bars, 25 μm . (G and H) Zoom images of a nucleus colabeled with PE-16 (G) and 3C5 (H), an antibody that detects interchromatin granules.



main patterns of proenkephalin distribution within the nucleus, using indirect immunofluorescent detection. Each of the 15 PE mAbs gives one of these staining patterns which we describe as fine punctate (*A* and *B*), coarse punctate (*C*), and speckled (*D*). (The coarse punctate and speckled staining patterns are also observed in cells fixed with methanol and acetone.) In asynchronous, exponentially growing cells, the coarse punctate and speckled (*C* and *D*) patterns are visible in all interphase nuclei. The fine punctate pattern (*A* and *B*) is usually visible in very few interphase cells (<5%) except under certain growth circumstances (as discussed later).

After treatment of the cells with 10 µg/ml actinomycin for 5 h (Fig. 1, *E* and *F*), in most cases the subnuclear organization was lost and replaced by a bright, diffuse distribution. Fig. 1 *E* illustrates the change in the coarse punctate and Fig. 1 *F* the change in the speckled pattern, which now become indistinguishable. Actinomycin, an inhibitor of transcription, is also known to have effects on nuclear architecture (Nickerson and Penman, 1992). Therefore, disruption of proenkephalin organization could reflect liberation of proenkephalin from nuclear substructures or complexes, to become diffusely liberated in the nucleoplasm.

To determine if any of the proenkephalin localization patterns correspond to identifiable nuclear subcompartments, double-label immunofluorescent staining was carried out with other antinuclear antibodies. The speckled pattern (Fig. 1, *D* and *G*) was found to colocalize with interchromatin granules (Fig. 1 *H*), detected with the antibody 3C5 (a gift from Bryan Turner, University of Birmingham, Birmingham, U.K.; Turner and Franchi, 1987). Fig. 1, *G* and *H*, shows images at higher magnification of a nucleus dual labeled with PE-16 (*G*) and 3C5 (*H*), an IgM mAb, followed with subclass-specific second antibodies. Neither pattern was observed with the incorrect second antibody, which excludes cross-reactivity. Colocalization was confirmed by computer overlay of the confocal images (not shown). Interchromatin granules represent a definable nuclear substructure, detectable by electron microscopy (Wassef, 1979). Proteins involved in RNA processing, including small nuclear ribonucleoproteins (snRNPs) and the splicing factor SC-35 (Spector et al., 1991), have also been localized to this compartment, but the exact nature of the events that occur here is unresolved. Indeed, proteins not involved in splicing, such as glutathione *S*-transferase, have an identical distribution (Bennett and Yeoman, 1985), so the compartment appears multifunctional. The coarse punctate (Fig. 1 *C*) pattern does not colocalize with 3C5 staining, which indicates that this distribution is distinct from interchromatin granules. We have failed to detect proenkephalin in coiled bodies (by costaining with anti-coilin antibodies from A. Lamond, European Molecular Biology Laboratory, Heidelberg, Germany), which is another domain implicated in the RNA processing pathway (Carmo-Fonseca et al., 1992).

Restricted Revelation of Proenkephalin Epitopes

We observed one of three predominant staining patterns with each monoclonal antibody. The speckled and coarse punctate patterns, seen in the majority of proliferating in-

terphase cells, appear not to overlap, because only the speckled pattern colocalizes with interchromatin granules. In a proliferating cell population, the fine punctate pattern characteristic of several antibodies is seen in <5% of cells. This indicates that, at least in proliferating cells, only limited regions of the proenkephalin molecule may be accessible to antibodies. To test this, we needed more information about the binding domains of the PE mAbs.

Proenkephalin epitopes recognized by these antibodies have now been fully characterized with phage display libraries incorporating randomly generated sequences and with synthetic peptides (Böttger et al., 1995). Fig. 2 summarizes this information and shows the core sequences of the epitopes recognized by the proenkephalin mAbs within the primary sequence of human proenkephalin. Additional data (Böttger et al., 1995) that define differences in antibody fine specificities indicate that the higher order structure of the protein may influence epitope recognition.

The antibodies PE-1, 13, 20, 21, 22, and 24 all reveal a fine punctate staining pattern (Fig. 1, *A* and *B*). These antibodies recognize the domains II and III (Fig. 2), which are adjacent on the proenkephalin molecule. Because the antibody PE-2, directed to a different site on proenkephalin (Fig. 2, *domain IV*), also reveals the same pattern, it is very likely that this is an authentic reflection of proenkephalin distribution. The revelation of domain I (recognized by PE-14, etc.) in this distribution remains possible because it may overlap with the speckled pattern. The fine punctate pattern of staining is normally evident in a very small percentage of proliferating, interphase cells (>5%) but is transiently revealed in response to external growth arrest and differentiation signals in up to 100% of cells (see later section).

A rather more complex picture emerges when analyzing the epitopes revealed in the coarse punctate and speckled distributions. The antibodies PE-14, 16, 17, 18, 19, and 25 are all associated with a speckled staining pattern (Fig. 1

MARFLTLC	TW	LLLLGPG	LLA	TVRAECS	QDC	ATCSYRL	VLRP	ADINFLAC	VVM	50	
ECEGKLP	SLK	IWETCKE	LLQ	LSKPELP	QDG	TSTLRENS	KP	EESHLLAK	KRY	100	
GGFMKRYGGF		MKKMDELY	PM	EPEEEANG	SE	ILAKRYGG	FM	KKDAEED	DSL	150	
		IV									
ANSSDILK	SEL	LETGDN	RERS	HHQD	GSDN	EE	KVSKRYGGFM	RGLKRSP	QLE	200	
		I					II	III			
DEAKELQ	KRY	GGFMRRV	GRP	EWWM	DYQ	KRY	GGFLKR	FAEA	LPSDEEG	ESY	250
SKEVPEM	EKR	YGGFM	RF							267	
I		PE-14, PE-15, PE-16, PE-17, PE-18, PE-19, PE-23, PE-25									
II		PE-13, PE-20, PE-21, PE-22, PE-24									
III		PE-1									
IV		PE-2									

Figure 2. Location of epitopes recognized by anti-proenkephalin mAbs within the primary sequence of proenkephalin. Epitopes were defined using phage display libraries and synthetic peptides. Four antigenic domains have been identified (*I* to *IV*); antibodies that recognize these domains are listed in the lower panel.

D) and all recognize the same epitope (Fig. 2, domain I). An exception is the antibody PE-15, which recognizes domain I but which gives a coarse punctate (Fig. 1 C) rather than a speckled pattern. Nevertheless, a comparison of the phage insert sequences (containing 6, 12, or 24 amino acids; Böttger et al., 1995) recognized by these antibodies shows that alteration of sequences that flank the DLL-KELL core epitope differentially constrains epitope recognition by PE-14 and PE-15, a situation that could be simulated in the nucleus by differences in the conformation or intermolecular interactions of proenkephalin in the two distributions. In addition, we have been able to provide additional evidence for the authenticity of these staining patterns with a differential extraction procedure (see later).

Intact Proenkephalin Precursor Protein Resides within the Nucleus

To confirm the existence of proenkephalin protein within the nucleus of 3T3 cells, nuclear isolates were made from cells in exponential growth phase by gentle homogenization in a nuclear isolation buffer followed by centrifugation through a sucrose cushion. Isolated nuclei were then immunoblotted against all 15 PE mAbs individually (Fig. 3 a, lanes 1 to 16; lane 15 no antibody control). A strong doublet or triplet of bands with apparent molecular weights of 34.5, 33, and 31.5 is detected with several of the antibodies, including antibodies directed to different epitopes, such as lanes 3, 4, and 11 (PE-13, 14, and 21). The reason some of

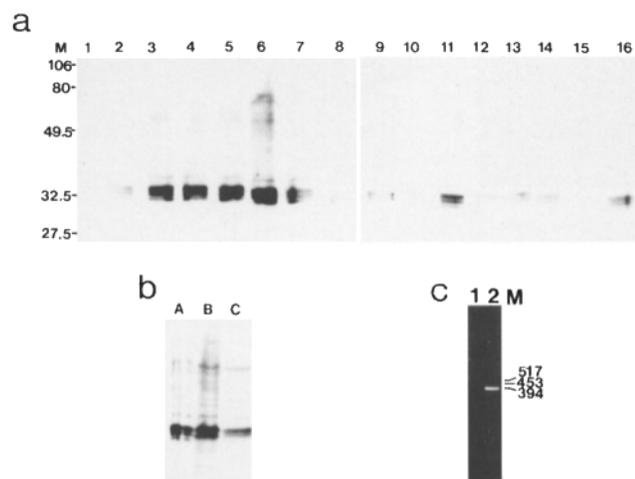


Figure 3. Confirmation of the existence of proenkephalin protein and mRNA in 3T3 cells. (a) Nuclei were isolated from proliferating 3T3 cells, then lysed and loaded onto a preparative SDS-polyacrylamide gel. After electroblotting, strips of membrane were incubated with individual PE mAbs, followed by peroxidase conjugated anti-mouse IgG before a chemiluminescence reaction (lanes 1-14: PE-1, 2, 13 through 24, respectively; lane 15, no primary antibody control; lane 16, PE-25; M, molecular mass marker track in kilodaltons). (b) Separate immunoblot analysis: lanes A-C compare transfected proenkephalin in PC12 cells (lane B) with proenkephalin in nuclear isolates (A) and total cell lysates (C) from 3T3 cells. Lanes A-C were immunostained with the same PE mAb. (c) Lane 2 shows PCR product obtained with mouse proenkephalin specific primers from 3T3 cell reverse transcripts; lane 1, nonmurine cell control; lane M, DNA size markers.

the antibodies work better in immunofluorescence than in immunoblotting may be because of a difference in the folding of nuclear proenkephalin on a blot compared with that in situ. In a separate immunoblot comparison (Fig. 3 b), 3T3 cell proenkephalin in nuclear isolates (lane A) comigrates with that in total cell lysates (C) and with human proenkephalin overexpressed in PC12 cells (lane B; Spruce et al., 1990). The observed molecular weight of nuclear proenkephalin is consistent with its existence in a predominantly uncleaved state (Spruce et al., 1988, 1990).

Proenkephalin mRNA Exists in 3T3 Cells

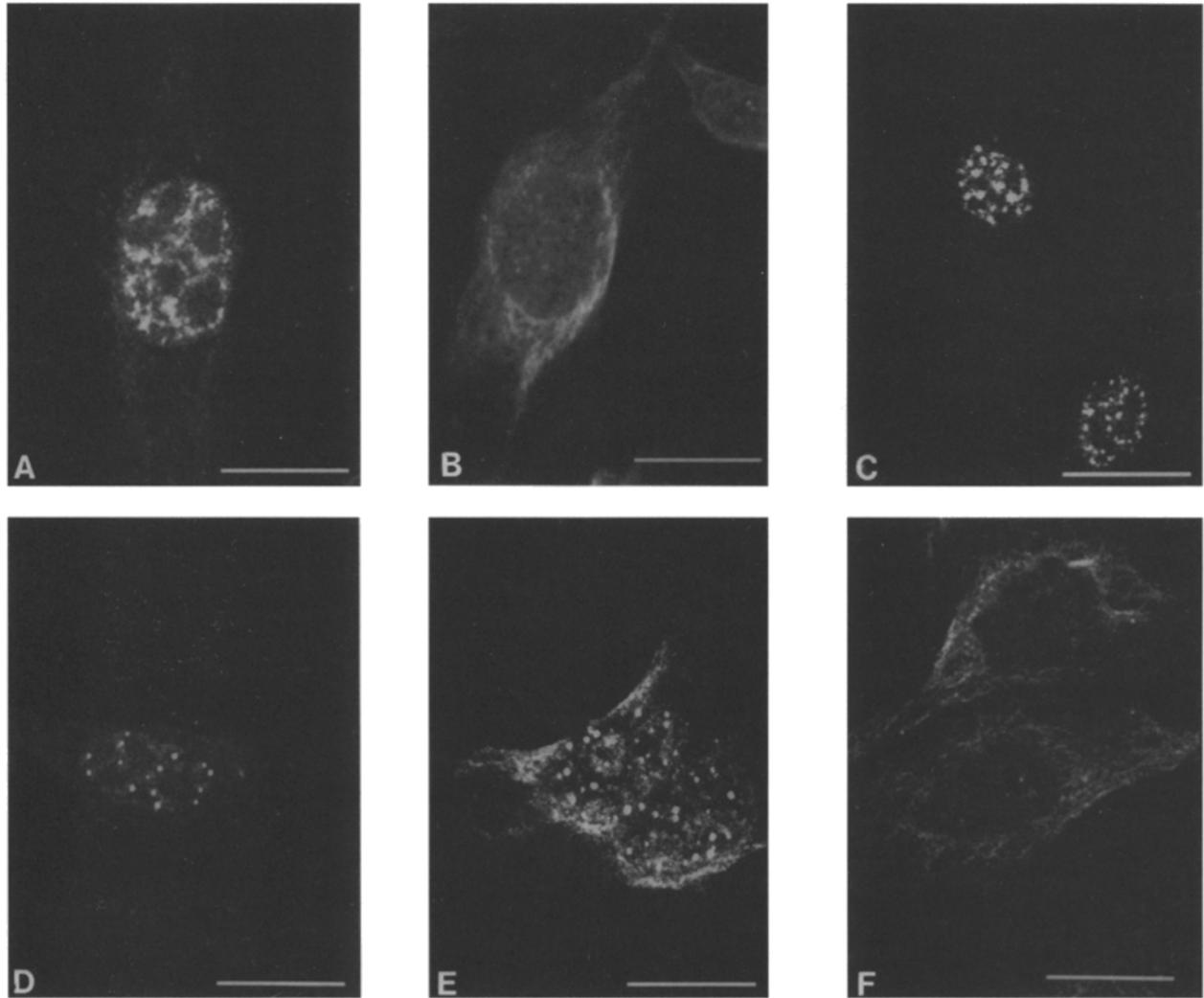
Proenkephalin mRNA has previously been demonstrated in transformed Fr3T3 cells (Matrisian et al., 1988). In non-transformed Swiss 3T3 cells, we observe a low abundance proenkephalin transcript on Northern blot analysis (not shown). To definitively show the existence of proenkephalin mRNA in 3T3 cells, we carried out PCR amplification of reverse transcripts synthesized from 3T3 cell mRNA. Primers specific to mouse proenkephalin were used: 5'-CTTCTTTCAAATCTGGGAGACCT-3' and 5'-ATACCTCTTGCTCATGTCTTCGTC-3'. A single PCR product of 392 base pairs was isolated (Fig. 3, c, lane 2) which was sequenced using the ds DNA cycle sequencing system (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, MD), incorporating both PCR primers in cycle sequencing reactions. The sequence of the PCR product was identical to that segment of the mouse proenkephalin sequence flanked by the two primers.

Subnuclear Fractionation Confirms Differential Compartmentalization of Nuclear Proenkephalin

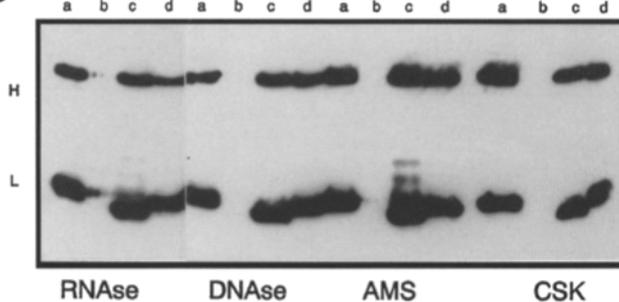
To validate our data further, we carried out subnuclear fractionation to determine if extraction of proenkephalin from particular distributions can be correlated with liberation of proenkephalin into the extraction supernatant. Pelleted cells and cells on tissue culture slides were extracted; supernatants from the former were subjected to nonradioactive immunoprecipitation with PE mAbs (Fig. 4 b) and compared with cells on slides fixed and immunostained postextraction (Fig. 4 a). Upper panels show nuclei with speckled (PE-16) staining (A) after preextraction with CSK buffer (see Methods), (B) after 0.25 M ammonium sulfate treatment (AMS),¹ and (C) after a combination of DNase and RNase treatment. The speckled pattern is resistant to CSK preextraction (A) and nuclease treatment (C) but is sensitive to salt (B). The disappearance of this pattern coincides with the appearance of immunoprecipitable proenkephalin in the supernatant after salt extraction (Fig. 4 b, AMS lane c). A product that resolves as a doublet migrating at approximately 34 and 31 kd is precipitated with PE-16 (AMS lane c). A different PE mAb was used to probe the blotted immunoprecipitation products. No products are precipitated from supernatant after CSK treatment (Fig. 4 b, CSK lane c), with control antibody (Fig. 4 b, AMS lane a, anti-p53 ascites) or without antibody (Fig. 4 b, AMS lane b, no ascites).

1. Abbreviations used in this paper: AMS, ammonium sulfate treatment; PCNA, proliferating cell nuclear antigen.

a



b



c

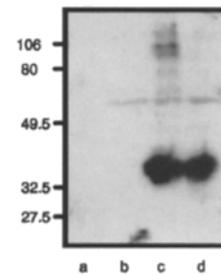


Figure 4. Proenkephalin exists in biochemically distinct subnuclear compartments. (a) Proenkephalin immunofluorescence staining (upper row, speckled pattern, PE-16; lower row, coarse punctate pattern, PE-15) was carried out on 3T3 cells after CSK preextraction (A and D), after salt (0.25 M ammonium sulfate, AMS, B and E), or after nuclease extraction (DNase followed by RNase, C and F). Bars, 25 μ m. (b) In parallel with immunolocalization after extraction, harvested 3T3 cells were sequentially extracted with CSK, salt (AMS), and DNase then RNase; after each extraction, soluble and insoluble fractions were separated by centrifugation and postextraction supernatants and then immunoprecipitated with PE mAbs or with control antibody. Immunoprecipitation products were immunoblotted against a PE mAb followed by an anti-mouse second antibody and then detected using ECL. H and L denote heavy and light chain immunoglobulin bands deriving from the precipitating antibody. In all cases, precipitating antibodies were as follows: lane a, anti-p53; lane b, no precipitating antibody; lane c, PE-16; and lane d, PE-15. (c) Cell pellets corresponding to postextraction supernatants in Fig. 4 b were analyzed by immunoblot to assess the proportion of proenkephalin remaining in the insoluble fraction after each extraction. Lane d, untreated pellet; lane c, pellet after CSK preextraction; lane b, pellet after salt extraction; and lane a, pellet after salt and nuclease extraction.

Fig. 4 *a*, panels *D*, *E*, and *F*, shows nuclei with the coarse punctate (PE-15) staining pattern (*D*) after CSK treatment, (*E*, binucleate cell) after AMS, and (*F*) after combined DNase and RNase treatment. This immunofluorescent staining pattern is resistant to CSK preextraction (*D*) and to salt extraction (*E*) but is sensitive to nuclease treatment (*F*). Sequential treatment of pelleted cells with AMS, then DNase and RNase, is associated with the appearance of PE-16-immunoprecipitable proenkephalin in the extraction supernatant, visible as a weak doublet of the same molecular size as that extracted by salt alone (Fig. 4 *b*, RNase lane *c*). PE-15, which recognizes the same core epitope as PE-16, fails to immunoprecipitate proenkephalin from any extraction supernatants (Fig. 4 *b*, lanes *d* throughout), which could be explained by recognition of different conformational forms of proenkephalin by PE-16 and PE-15 because of differential constraints on revelation of the core epitope. When subjected to nuclease treatments in the reverse order or to RNase alone, the coarse punctate proenkephalin staining is only partially diminished; DNase when free of RNase has no effect. We attribute this to a cooperative effect of DNase, perhaps improving accessibility for RNase extraction.

To assess the proportion of proenkephalin remaining in the insoluble fraction following extraction, cell pellets were immunoblotted against PE mAb (Fig. 4 *c*). Lanes *d* (untreated) and *c* (CSK treated) show a prominent PE-immunoreactive band that disappears after sequential salt (lane *b*) and nuclease (lane *a*) extraction. Although proenkephalin in the cell pellet after salt extraction and before nuclease extraction is below the limit of detection by immunoblot analysis, a small quantity of proenkephalin is liberated into the supernatant after sequential salt and nuclease extraction; this is consistent with the persistence of the coarse punctate staining pattern after salt extraction. From these data we conclude that the majority of nuclear proenkephalin resides in a salt-extractable compartment; a smaller proportion is in a nuclease-extractable compartment, which corresponds to the coarse punctate distribution that varies in intensity between cells.

We have not yet examined the behavior of the fine punctate staining pattern under these extraction conditions because it is usually visible in only a very small percentage of cells except under special circumstances (see later section). However, the pattern is diminished and sometimes abolished when cells have been subjected to methanol. This provides evidence for a further difference in the association of proenkephalin with the nuclear subarchitecture when proenkephalin is in this distribution, since it contrasts with the stability of the speckled and coarse punctate staining distributions when cells are subjected to methanol alone or in combination with acetone (not shown).

Change in the Revelation of Proenkephalin Antigenic Domains in the Transition to Growth Arrest

Fibroblasts exit the cell cycle, becoming diverted to a quiescent or G₀ state, when deprived of serum or growth factors, or when maintained at confluency to induce contact inhibition. We examined the behavior of nuclear proenkephalin in both of these states of growth arrest.

When 3T3 cells are serum deprived, there is variation in the subnuclear response of proenkephalin. In general,

when cells are serum deprived at high but not low density, there is a reduction in nuclear proenkephalin immunofluorescence. Serum deprivation, particularly when in combination with high density culture conditions, increases the tendency of 3T3 cells to spontaneously transform. Therefore, to exclude these effects, we chose to examine MRC-5 human embryonic lung fibroblasts which are known to quiesce more reliably in response to serum deprivation. An asynchronous population of MRC-5 cells will exit into G₀ ~18–22 h after serum withdrawal, the time taken to traverse the cell cycle.

We examined proenkephalin immunofluorescence in MRC-5 cells at various times after transfer from 10 to 0.2% FCS, illustrated in Fig. 5. Cells were immunostained at intervals with anti-proenkephalin mAbs and with antibodies to proliferating cell nuclear antigen (PCNA) (provided by N. Waseem and D. Lane, University of Dundee, Dundee, U.K.; Waseem and Lane, 1990), to confirm their exit from the cell cycle. PCNA, an accessory protein for DNA polymerase delta, is localized to DNA replication complexes in S phase of the cell cycle. This is revealed by a punctate distribution of the protein; after liberation from replication complexes, PCNA becomes diffusely distributed in the nucleoplasm (Bravo and Macdonald-Bravo, 1987). Because PCNA has a long half-life within the nucleus, only the punctate staining pattern is a reliable indicator of cell proliferation.

Fig. 5, column *A*, shows proenkephalin immunostaining before serum withdrawal; column *B*, cells 4 to 6 h after transfer to 0.2% FCS; *C*, cells after 24 h of serum deprivation. As in 3T3 cells, the speckled stain (*A*, upper row, detected with PE-18) is seen in all interphase nuclei in an asynchronous cell population. The fine punctate stain (*A*, second row, PE-2) is rarely present in an exponentially growing population, again as in 3T3 cells. The coarse punctate pattern, detected with PE-15 in 3T3 cells, is not seen. This may reflect either interspecies variation in subnuclear compartments or a difference in the growth and/or differentiation state of these two fibroblast cell lines.

4 to 6 h after serum withdrawal (Fig. 5, column *B*), the speckled stain is unchanged from baseline. However, there is a dramatic change in the fine punctate stain, detected with antibodies directed to domains II, III, and IV. Barely visible at baseline, it is now present in up to 100% of cells (Fig. 5, column *B*, middle row). At this stage, the cells are still traversing the cell cycle; S-phase cells with punctate PCNA stain (*B*, lower row) are present, together with mitotic cells (a metaphase cell is seen in *B*, detected with PE-18; proenkephalin immunostaining changes at mitosis, becoming bright and diffuse, and is excluded from the condensed chromosomes, seen in this cell aligned on the metaphase plate). The revelation of proenkephalin epitopes in a fine punctate distribution is no longer seen beyond 8 h.

24 h after serum withdrawal (Fig. 5, column *C*), the cells have exited the cell cycle; punctate PCNA staining is absent, and no mitotic cells are seen. At this stage, the speckled nuclear stain appears unchanged (upper row) and the fine punctate stain remains absent (middle row).

The prompt unmasking of proenkephalin antigenic domains II, III, and IV upon mitogenic factor withdrawal may reflect receipt of a growth arrest signal from the extracellular environment. To test whether this phenomenon

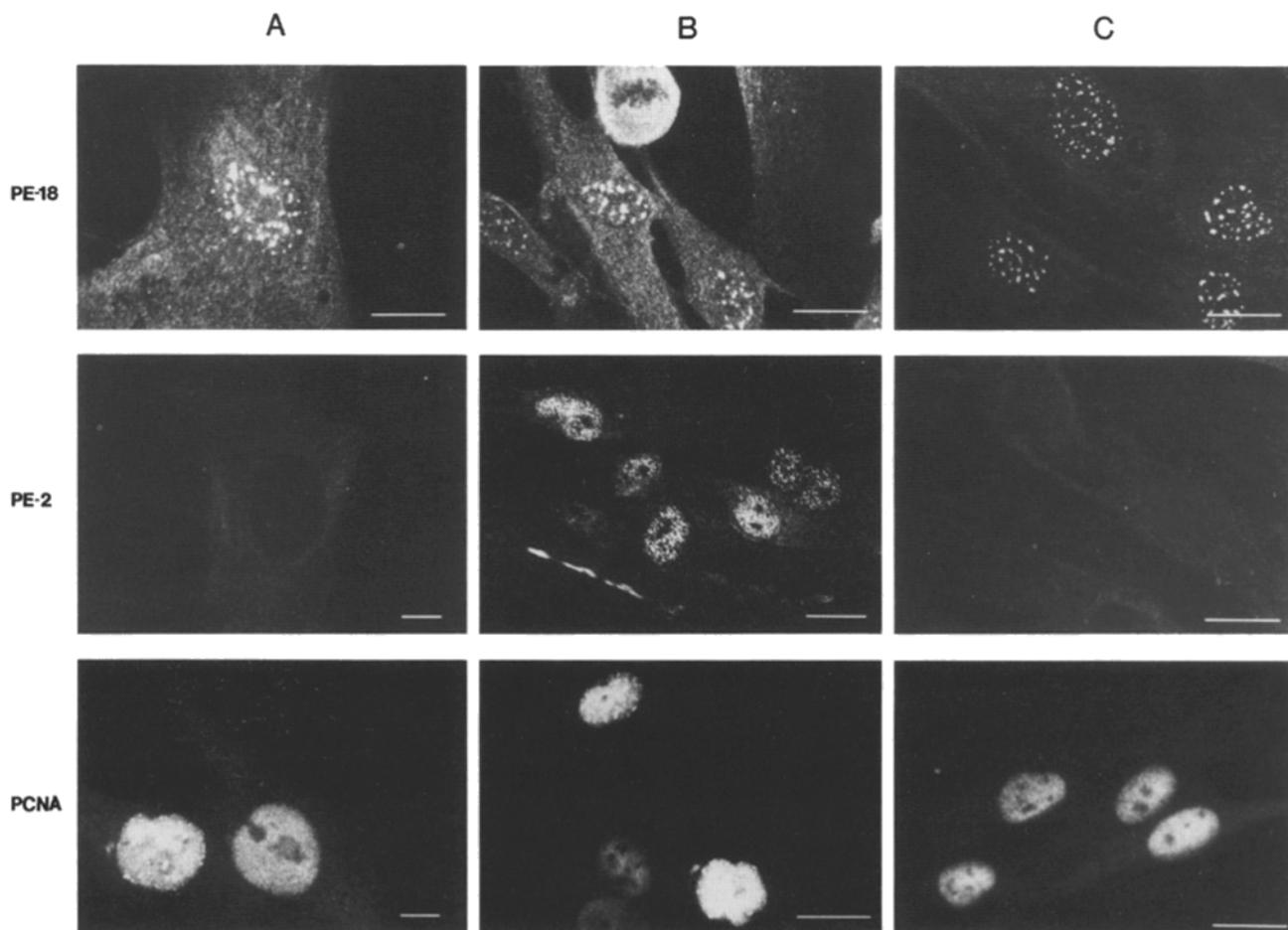


Figure 5. Mitogen withdrawal induces an early and transient response in nuclear proenkephalin. MRC-5 cells were seeded sparsely in 10% FCS, then transferred to 0.2% FCS after 24 h. Cells were fixed and immunostained with PE or PCNA mAbs (*A*) before and (*B* and *C*) at intervals after serum withdrawal. Column *B* represents cells 4 to 6 h after serum withdrawal, before cell cycle exit. Bars, 10 μ m (*A*, lower two panels) or 25 μ m (remaining panels).

was also present after another stimulus to growth arrest, we examined 3T3 cells rendered contact inhibited by culture at high density in the presence of 10% FCS. At \sim 2 d after plating, when cells have reached confluence but are not yet arrested, revelation of proenkephalin antigenic domains II, III, and IV in a fine punctate distribution is again seen in the vast majority of cells, and again the response is transient (not shown). If 3T3 cells are maintained at high density, there is down-regulation of nuclear proenkephalin staining.

The changes in nuclear proenkephalin immunofluorescent staining patterns in the transition to growth arrest occur in the absence of any detectable change in the amount or molecular forms of proenkephalin protein compared with that extracted from nuclei of proliferating cells (Fig. 3). Thus, the changes in staining must reflect differential unmasking and concealment of proenkephalin epitopes, possibly combined with subnuclear redistribution, during the induction of growth arrest.

Dynamic Subnuclear Reorganization of Proenkephalin in Myoblasts Undergoing Differentiation

In undifferentiated rodent myoblasts (C2C12 cells; Yaffe

and Saxel, 1977, subcloned by H. Blau), proenkephalin exists in the nucleus in coarse punctate (Fig. 6, *upper row, D1*) and speckled distributions (not shown), as in embryonic fibroblasts. Again, the fine punctate pattern revealed with antibodies directed to domains II, III, and IV is seen very rarely in undifferentiated cells (Fig. 6, *lower row, D1*). However, if C2C12 cells are induced to commence differentiation either by growth to confluence or by transfer from FCS to 1% donor horse serum, domains II, III, and IV in a fine punctate distribution are transiently revealed in up to 100% of cells (Fig. 6, *D4*). At this stage (between days 3 and 5) the cells have not yet fused to form myotubes, and they are proliferating asynchronously. As in fibroblasts in transition to growth arrest, the revelation of multiple proenkephalin epitopes in myoblasts early in their differentiation program is also transient (Fig. 6, *D7* shows an absence of PE-2 staining). However, domains II, III, and IV are revealed again at a later stage of differentiation (Fig. 6, *D10, lower row*) when the cells are fusing to form multinucleated myotubes. In multinucleate myotubes, the revelation of domain I in a punctate distribution changes to become brighter and more diffuse (Fig. 6, *D10, upper row*). Throughout these stages, levels of proenkephalin protein detected by immunoblotting remain ap-

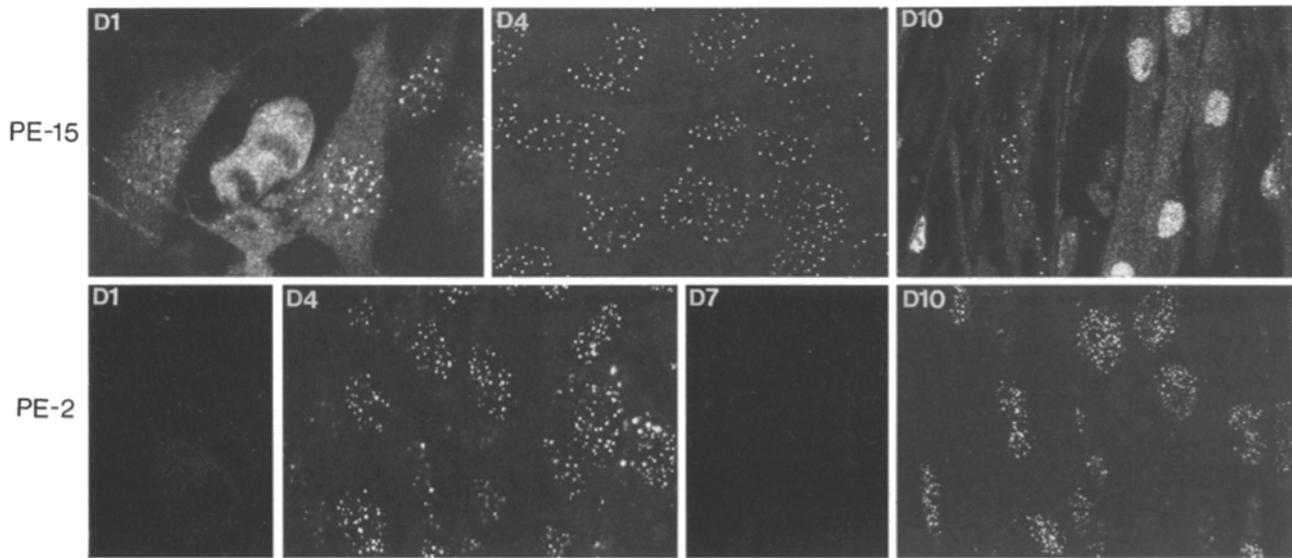


Figure 6. Change in nuclear proenkephalin staining during myoblast differentiation. C2C12 cells were transferred from FCS-containing medium to medium with 1% donor horse serum. Cells were fixed and immunostained with PE mAbs at intervals (days 1, 4, 7, and 10) during differentiation. On day 4 (D4), multiple proenkephalin epitopes are transiently revealed in up to 100% of cells.

parently unchanged. Thus, as in fibroblasts in transition to growth arrest, there is dynamic unmasking and concealment of proenkephalin epitopes, and possibly subnuclear redistribution, as myoblast differentiation proceeds.

Proenkephalin Devoid of a Signal Peptide Sequence Is Targeted to the Nucleus in Transiently Transfected COS Cells

To determine which regions of the proenkephalin molecule may be involved in nuclear targeting, we have begun a series of mutagenesis experiments. In COS cells transiently transfected with nonmutated proenkephalin cDNA, the transfected gene product is localized exclusively in the cytoplasm (Fig. 7, *top row*). Immunofluorescent staining was performed with antibodies to two different epitopes (domains II or III, and domain I) to confirm its authenticity. In contrast, if site-directed mutagenesis is used to change the normal ATG initiation codon to an ATC codon from which translation cannot be initiated, efficient expression of the transfected gene is seen but the gene product is now targeted to the nucleus as well as to the cytoplasm (Fig. 7, *second row*). From this we conclude that, in the absence of the normal AUG initiation codon, translation can be initiated at another codon to produce a form of proenkephalin with nuclear and cytoplasmic fates.

It is known that FGF molecules can be translated from upstream CUG initiation codons; this leads to the production of forms of FGF with both nuclear and secretory pathway destinies (Bügler et al., 1991; Kiefer et al., 1994). The proenkephalin gene possesses two upstream CTG codons, which are in translational reading frame with the coding sequence. To test if translation could be initiated from one or both of these CTG codons, mutant cDNAs were generated in which one or the other of the CTG codons, both CTG codons, or both CTG codons together

with the first ATG codon were changed and then transfected separately into COS cells. In all cases, translation remained efficient, and a proportion of the transfected gene product was translocated to the nucleus; that is, the result was indistinguishable from mutation of the first ATG alone (Fig. 7, *second row*). This indicates that proenkephalin can be translated from one or more downstream initiation codons.

Additional ATG codons within proenkephalin representing potential sites of alternative initiation are located downstream from the signal peptide sequence. This suggested to us that absence of the signal peptide sequence may cause the molecule to circumvent the secretory pathway and thereby permit nuclear transfer. To test this we used a PCR approach to delete the signal sequence but retain the normal ATG initiation codon. The product of such a construct is also targeted to the nucleus (Fig. 7, *third row*, ATG Δ sig). This indicates that removal of the signal peptide is sufficient to confer a nuclear fate to proenkephalin.

Discussion

Subnuclear Revelation of Proenkephalin Depends on Growth Arrest and Differentiation Signals

In this paper we show that proenkephalin is a nuclear protein in fibroblast and myoblast cell lines and that the revelation of antigenic domains is dependent on signals from the extracellular growth environment.

In nuclei of proliferating interphase Swiss 3T3 and MRC-5 cells, and in undifferentiated C2C12 myoblast cells, the exposure of antigenic domains on proenkephalin is restricted. This may be a result of either conformational constraints or complex formation with other nuclear proteins, which may regulate the exposure or interactions of

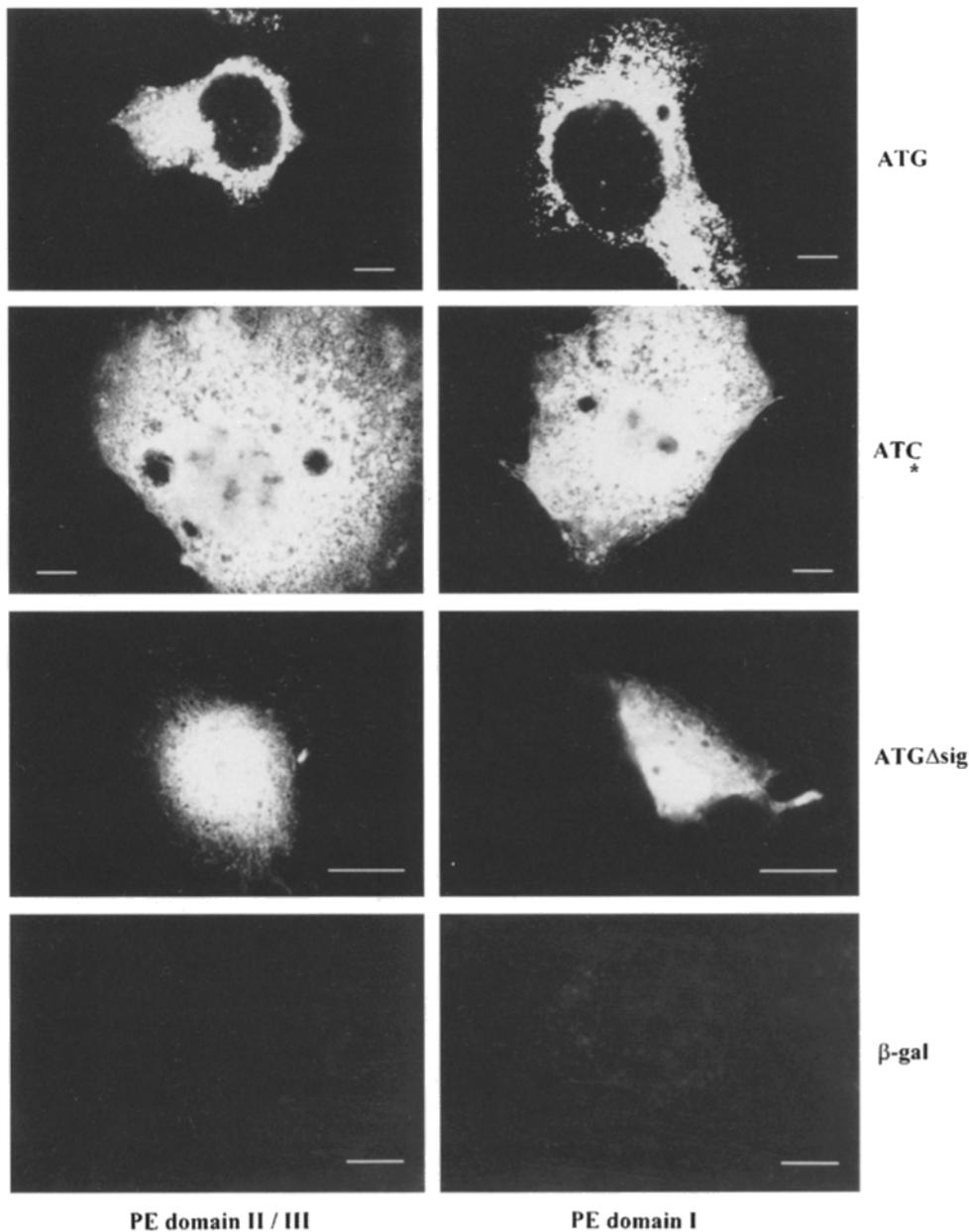


Figure 7. Nuclear targeting of mutated proenkephalin transiently transfected into COS cells. Nonmutated and mutated proenkephalin cDNAs were transiently transfected into COS cells; cellular localization of transfected proenkephalin was confirmed by indirect immunofluorescence staining with PE mAbs to different epitopes (domain II or III, left-hand column; domain I, right-hand column). The upper row shows the product of nonmutated proenkephalin (ATG); the second row, proenkephalin mutated at the first ATG codon (ATC); the third row, proenkephalin mutated to remove the signal peptide sequence (ATG Δ sig); and the bottom row, control cells transfected with β -galactosidase expression vector alone (β -gal). Bars, 25 μ m (third row) and 10 μ m (remaining rows).

functional domains. It may also serve to prevent enzymatic cleavage of proenkephalin, a molecule ordinarily highly susceptible to proteolytic breakdown.

We observed low levels of proenkephalin mRNA in nontransformed 3T3 cells. Higher levels of proenkephalin mRNA have previously been described to exist in transformed 3T3 cells (Matrisian et al., 1988). We have preliminary data that proenkephalin may relocate to the cytoplasm when 3T3 cells undergo spontaneous transformation, which could explain the higher mRNA levels if this is associated with a shorter protein half-life. However, the apparent revelation of cytoplasmic proenkephalin, such as after salt extraction (Fig. 4 *a*, panels *B* and *E*) and in MRC-5 cells (Fig. 5), suggests that proenkephalin exists in the cytoplasm as well as in the nucleus of nontransformed cells but that it is more often masked in the cytoplasm of nontransformed cells.

In the transition to growth arrest, antigenic domains on

nuclear proenkephalin are transiently unmasked in up to 100% of cells; this coincides with newly established cell-cell contact in 3T3 cells and occurs within 4 to 6 h of mitogen withdrawal in MRC-5 cells. The rapidity of the response, together with the existence of S-phase and mitotic cells at this time, is therefore inconsistent with synchronization at a G1/G0 boundary. The same phenomenon is also observed in rodent myoblast (C2C12) cells early in their differentiation program. Together, these data imply that nuclear proenkephalin can acknowledge signals that mediate growth arrest and differentiation in a time window apparently independent of cell cycle stage.

Mechanism of Nuclear Targeting

We have shown that removal of the signal peptide is sufficient to confer a nuclear fate to transiently transfected proenkephalin in COS cells. The similarity of the molecu-

lar size between nuclear and secretory proenkephalin, from which the signal peptide will have been cleaved during translocation across the endoplasmic reticulum membrane while translation is still proceeding, is consistent with derivation of the nuclear form from a primary translation product that has never possessed a signal sequence. How might a primary translation product that lacks a signal sequence arise *in vivo*? It is known that a population of cells in the rat forebrain contains proenkephalin primary transcripts initiated from an alternate site in intron A and lacks exon 2-containing transcripts (Brooks et al., 1993). A rat proenkephalin transcript that lacks exon 2 (equivalent to exon 3 in the human gene) would direct a protein devoid of a signal sequence. It is possible, therefore, that alternate transcription initiation, alternate splicing, or a combination of both could lead to a primary translation product devoid of a signal peptide, which may then be capable of nuclear translocation.

We still, however, cannot definitively exclude the additional possibility that translation initiation from one of the upstream in-frame CUG codons in proenkephalin may also occur, as with FGF family members (Bügler et al., 1991; Kiefer et al., 1994). Furthermore, it is well established that secreted growth factors internalized by target cells can be translocated to the interior of the nucleus as well as to the nuclear membrane (Yankner and Shooter, 1979; Johnson et al., 1980; Rakowicz-Szulczynska et al., 1986). Nuclear translocation of occupied polypeptide hormone (insulin) cell surface receptors has also been described (Podlecki et al., 1987). It therefore remains possible that re-uptake of a secreted form of proenkephalin, possibly remaining bound to cell surface receptors, may be an additional mechanism of nuclear transfer; however, nuclear targeting of proenkephalin mutated to remove the signal sequence, which therefore cannot gain access to the secretory pathway, demonstrates that "intracrine" nuclear transfer can occur.

Why Should a Neuropeptide Precursor Have Such Diverse Roles?

We have shown that proenkephalin exists as a nuclear protein integrated with pathways that mediate growth arrest and differentiation signals. In the nervous system, enkephalin peptides act as neurotransmitters and neuromodulators. Other proteins, most notably the crystallins, have functionally diverse roles subserved by common protein domains (Wistow et al., 1987), which may reflect evolutionary molecular economization ("gene sharing"). A theoretical paper has indicated the possibility that the opioid precursors prodynorphin and proenkephalin may be DNA-binding proteins on the basis of their possession of zinc finger and helix-loop-helix domains, together with amino acid homology with *hunchback*, *lxl-1*, and related proteins (Bakalkin et al., 1991). This molecular possibility will be interesting to explore.

A negative consequence of gene sharing is that it may impose an unfavorable evolutionary constraint in that adaptive change in one function should not be detrimental to the other(s). However, obligate coupling of adaptive change in the two or more functions may be advantageous if a common signaling or effector pathway immediately

downstream of the multifunctional protein is involved. It is possible that the diverse roles of proenkephalin are a result of coevolution with common signaling mechanisms to subserve different functions according to subcellular location or cell type. Phosphoinositol hydrolysis occurs within the nucleus of Swiss 3T3 cells (Divecha et al., 1991). This opens up the possibility that receptor-coupled signals generated either within the nucleus or at the nucleocytoplasmic interface could be transduced in a similar way to signals at the cell surface. Opioid binding in association with a nuclear fraction from developing cerebellum has been reported (Zagon et al., 1991). Belcheva et al. (1993) have shown the existence of G protein-coupled delta opioid receptors in highly purified nuclear membrane fractions from a neurohybrid cell line; uncoupled opioid binding sites that may be chromatin-associated have also been detected. We have shown that an opioid precursor, proenkephalin, exists in the nucleus in an apparently unprocessed form in that we fail to detect proteolytic intermediates with our PE mAbs (Spruce et al., 1988). It remains possible that proenkephalin may undergo localized proteolytic cleavage to generate opioid ligands at specific subnuclear sites of activity, or proenkephalin could be acting independently of opioid receptors. Opiate binding at the cell surface has also been linked to proliferation and differentiation events (Hauser et al., 1989, 1992). Taken together, these results suggest that proenkephalin and opioid peptides could be involved in growth control in the nucleus and at the cell surface. There is evidence that nonopioid neuropeptides also have roles in cell proliferation (Nilsson et al., 1985; Haegerstrand et al., 1990), indicating that there may have been evolutionary pressure to coordinate neuropeptide function with growth control.

We thank Deborah Dewar for performing the site-directed mutagenesis; Volker Böttger, Frances Fuller-Pace, Angus Lamond, David Lane, and Bryan Turner for helpful advice; and A. Lamond, D. Lane and B. Turner for antibodies.

This work was supported by the Medical Research Council (UK) and the Wellcome Trust. B. A. Spruce is a Wellcome Senior Research Fellow in Clinical Science.

Received for publication 1 February 1995 and in revised form 1 May 1995.

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